LESSON 30:
TRANSGENIC PLANTS APPLICATIONS

Introduction

By using a variety of techniques, it has become possible to transform plants with foreign genes. Expression of foreign genes in plants makes it possible to produce a very wide range of new plant varieties. Transgenic plants have been developed to be resistant to a range of environmental stresses, including insects, viruses, herbicides, pathogens, and salt stress; to have flowers with modified colours; to a modified nutritional content, including modifications in amino acids, lipids, discolouration, and sweetness.

Virus-Resistant plants

Plant viruses often cause considerable damage and significantly reduce yield. Breeding for disease resistance is the best method to protect plants from viral and other infections. Recently, scientists have used the techniques of genetic engineering to develop virus-resistant transgenic plants. These methods used "immunization" with viral coat protein genes, other viral genes, or viral gene antisense sequences to confer resistance. .

Potato is one of the most important food crop after cereals and pulses. It is very difficult to improve potato through breeding techniques as it is a tetraploid. Most of the cultivars are susceptible to various diseases caused by fungi, nematodes, and viruses. Potatoes are vegetatively propagated, therefore, seed material (tubers) for planting must be virus-free. Potatoes suffer from three important virus diseases called Photo virus X, Photo virusY, (PVX, PVY) and potato leafroll virus.

The phenomenon of 'cross-protection' or immunization of plant is not clearly understood. It is similar to immunization of human-being for bacterial diseases. When a plant is inoculated with a mild strain of a virus that causes almost no disease symptoms, it will be protected against a virulent form of the virus. This cross-protection is in some way related to the synthesis of the coat protein by the plant cell. When virus infects plant cell, a plant cell starts synthesizing coat proteins instead of its own proteins.

In 1986, Roger Beachy and colleagues at Washington University introduced the gene that encodes the coat protein of TMV into tobacco plants, resultantly each and every cell of the transgenic plant start producing coat protein. These plants showed considerable resistance to infection by TMV. The virus was unable to multiply rapidly in the cells already containing some coat protein. Therefore, the number of virus particles per cell remained low in transgenic plants as compared to normal control plants.

Scientists at Mogen International in the Netherlands used the same approach to make potatoes resistant to PVX. The gene encoding the coat protein of PVX was introduced into two cultivars. The transgenics showed 100 times less virus particles as compared to control plants after two weeks of inoculation. The yield performance of most cultivars was same but potatoes produced were elongated. The viral coat protein gene approach has been used to transfer tolerance to a number of different plant viruses (Table 1.). Using this approach, researchers have developed virus-resistant transgenic plants for a number of different crops. Although complete protection is not usually achieved, high levels of virus-resistance have been reported. Moreover, a coat protein gene from one virus sometimes provides tolerance to a number of unrelated viruses.

In both eukaryotes and prokaryotes, an RNA molecule that is complementary to a normal gene transcript (mRNA) is called antisense RNA. The mRNA, being translatable, is considered to be a sense RNA. The presence of antisenses RNA can decrease the synthesis of the gene product by forming a duplex molecule with the normal sense mRNA, thereby, preventing it from being translated. The antisense RNA-mRNA duplex is also rapidly degraded, a response that diminishes the amount of that particular mRNA in the cell. Therefore, in principle it should be possible to prevent plant viruses from replicating and subsequently damaging plant tissues by creating transgenic plants that synthesize antisense RNA that is complementary to virus coat protein mRNA.

The Ti binary vector system was used to transfer both protein-producing sense and anti-sense RNA-producing cDNA sequences to separate tobacco cells, from which transgenic plants were regenerated. The transgenic tobacco plants that expressed the cucumber mosaic virus (cuMV) coat protein were protected from viral particle accumulation and did not show symptoms of viral infection, irrespective of whether the inoculum of the challenge virus was high or low. However, the transgenic tobacco plants expressing the CuMV coat protein antisense RNA were protected only when the concentration of the challenge virus in the inoculum was low. Therefore, this approach is not successful when virus infection is high.

Table 1. Virus-resistant transgenic plants developed that contain cloned viral coat proteins (genes)

Plant species	*Virus that provided the coat protein gene*
Nicotiana benthamiana	Plum pox virus, watermelon mosaic virus 2.
Papaya, tobacco	Papaya ring spot virus
Potato	Potato virus (PV)X, PVY, PVS,
Rice	Rice stripe virus
Tobacco	Soybean mosaic virus, Tobacco streak virus, Tomato spotted wilt virus, PVX.
Tobacco, alfalfa, tomato	Alfalfa mosaic virus
Tobacco, cucumber	Cucumber mosaic virus
Tomato	Tomato mosaic virus

Herbicide-Resistant Plants

Certain herbicides can be used as pre-emergence herbicides to kill weeds before the crops are planted. If crop plants are resistant to these chemicals then they can be used with crop plants (post emergence). By understanding the mechanism of action of these herbicides and development of resistance by certain bacteria to such chemicals can provide clone for developing herbicide tolerant plants. Some plants or bacteria are resistant

because they have an enzyme that detoxifies the herbicide. In other words, they possess a gene for this action. Transfer of this gene to a crop plant should protect the crop plant by same action or mechanism. Some plants or bacteria become resistant to herbicide because of mutation in the target enzyme (or gene), and because of this change they are no more sensitive to herbicide or are not damaged by herbicides. The enzyme can work in presence of herbicide. Therefore, the detoxifying mechanism or change in affected enzyme can make the organism herbicide tolerant.

Glyphosate (a herbicide) acts by inhibiting one of the enzymes that is necessary for the synthesis of amino acids in the chloroplast. Glyphosate, initially produced and marketed by Monsanto under the trade name 'Roundup', is a widely used non-selective herbicide, it effectively kills 76 of the world's 78 worst weed species.

Scientists at Monsanto isolated a gene for an enzyme involved in amino acid biosynthesis enzyme EPSP-synthase (5-enol pyruvinyl shikimate 3-phosphate synthase) from resistant E.coli bacteria. They modified the gene in such a way that it could be expressed in plants, and then transferred it to plants e.g., tobacco, tomato and soybean. Expression of bacterial gene in plant required a control region that would direct the expression at the gene in the plant (because bacterial control regions do not work in plants). In addition to this, the gene had to be modified in such a way that the enzyme, which is synthesized in cytoplasm, would be transported to chloroplast. This is important that when gene of prokaryotic origin is used, the product should be transported to right cellular compartment in the plant. This should not affect the quality or quantity of yield. The gene has been successfully transferred in soybean, where the plants showed resistance without change in yield.

Phosphinothricin is a herbicide that acts by inhibiting another enzyme necessary for amino acid biosynthesis (glutamine synthetase) and nitrogen metabolism. This enzyme converts ammonia to glutamate. Inhibiting the activity of this enzyme leads to rapid accumulation of ammonia within the plant cells. Higher concentrations of ammonia are toxic to the cell. Phosphinothricin produced and marketed by Hoechst AG under the trade name 'Basta', is also a very effective non-selective herbicide. This product is related to an antibiotic that is also a herbicide, produced by the fungus Streptomyces hygroscopicus. Scientists at Plant Genetic systems, Belgium obtained a gene from this fungus that encodes an enzyme that converts phosphinothricin to a non-herbicidal derivative by combining it with a cell metabolite. This gene, known as bar-gene, has been transferred in tobacco and potato, where it is expressed showing herbicide tolerance in these plants. The yield performance of the plants remained unchanged.

Herbicides are simply chemical compounds that kill or inhibit the growth of plants. without deleterious effects on animals. Herbicides usually inhibit processes that are unique to plants, e.g., photo-synthesis. Mostly herbicides act as inhibitors of essential enzyme reactions. Any change which can reduce the inhibitory effect of herbicide, will provide increased herbicide tolerance.

Glyphosate acts by inhibiting the enzyme 5-enol pyruvinyl-shikimate 3-phosphate synthase (EPSP synthase), an essential enzyme in the biosynthesis of the aromatic amino acids, tysosine, phenylalanine and tryptophan. These are essential components in the diets of higher animals since the enzymes that catalyze the biosynthesis of these amino acids are not present in higher animals. Therefore, higher animals do not contain EPSP synthase, and are not affected by glyphosate.

Glyphosate does inhibit the EPSP synthase of micro-organisms as well as those of plants. Selection of organisms is made on inhibitory concentrations of herbicide by growing them in presence of herbicide. This way researchers isolated glyphosate tolerant mutant of Salmonella typhimurium, Aerobacter aerogenes and Escherichia coli. In bacteria, EPSP synthase is encoded by the aeroA gene. When aeroA genes (with plant promoter and adenylation signals) were transferred in plants, the transgenic plants showed increased tolerance to glyphosate. In plants, aromatic amino acids are synthesized in chloroplasts, but gene for EPSP is localized in nucleus. Therefore, a protein is attached to EPSP synthase, which translocates the EPSP synthase into chloroplast, where the protein is removed by clearage. It has been shown that the petunia transit peptide will target the E.coli aeroA gene-product into tobacco chloroplasts and will impart glyphosate tolerance.

In another method, glyphosate-tolerant plants have also been produced by using an EPSP synthase cDNA isolated from a glyphosate tolerant petunia cell culture line. Such lines can be selected by growing cells on medium containing increasing concentration of selection factor, e.g., glyphosate. In this cell line, tolerance resulted from amplification (an increase in copy number) of the EPSP synthase gene, resulting in ovvr-production of EPSP synthase in these cells.

The EPSP synthase cDNA isolated from this cell line was joined to the CaMV 35s promoter and to the Ti nos- polyadenylation signal. The strong CaMV 35s promoter (from cauliflower mosaic virus) results in over production of EPSP synthase. When this chimeric (35s + EPSP synthase + nos) gene was introduced into petunia plants on a Ti vector, the transgenic developed were tolerant to the four times higher concentration, which kills control plants.

The other examples of herbicide resistant plants are given in the Table 2. It is evident from these examples that a resistant factor is developed based on mode of action of herbicide, by modifying or over producing the target product.

Table 2. Gene- based herbicide resistance in plants

Herbicides	*Mode of development of herbicide resistance*
Triazines	Resistance is due to an alteration in the *psbA* gene, which codes for the target of this herbicide, chloroplast protein D1.
Sulphonylureas	Genes encoding resistant versions of the enzyme acetolactate synthetase have been introduced into poplar, canola, flax and rice.
Glyphosate	Resistance is from overproduction of EPSPS, the target of this herbicide.
Bromoxynil	Resistance to this photosystem II inhibitor has been created by transforming tobacco and cotton plants with a bacterial nitrilase gene, which encodes an enzyme that degrades this herbicide.
Phenoxy carboxylic acids (e.g., 2, 4-D and 2, 4, 5-T)	Resistant cotton and tobacco plants have been created by transformation with the *tfdA* gene from Alcaligenes, which encodes a dioxygenase that degrades this herbicide.
Glufosinate (Phosphinothricin)	Over 200 different plants have been transformed with either the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *S. viridochromogenes*. The phosphinothricin acetyltransferase that these genes encode, detoxifies this herbicide.
Cyanamide	Resistant tobacco plants were produced when a cyanamide hydratase gene from the fungus *Myrothecium verrucaria* was introduced. The enzyme encoded by this gene converts cyanamide to urea.

Bt gene and Toxin (Bacillus thuringiensis)

Several species of bacteria produce proteins in abundance. When insect larvae ingest these bacteria with food, proteins kill larvae. The most widely studied of these bacteria is Bacillus thuringiensis or Bt in short. This species lives all over the world. When these bacteria form spores, they also form a large crystal like structure, in the bacterial cytoplasm that is made out of protein. This bacterium comprises a number of different strains and subspecies, each of which produces a different protein (toxin) that can kill certain specific insects (see Table 3.).

Table 3. Insecticidal toxins from some strains of B. thuringiensis

Strain	*Toxin class*	*Protoxin size (KDa)*	*Target species*
Berliner	Cry I	130-140	Lepidoptera
Kurstaki KTO, HD-1	Cry I	130-140	Lepidoptera
Kurstaki HD-1	Cry II	71	Lepidoptera, Diptera
tenebrionis (sandiego)	Cry III	66-73	Coleoptera
israelensis	Cry IV	68	Diptera

One of the proteins in the crystallike structure is called the Bt-protoxin. When insect larvae eat the bacterial cells along with leaves, the spores and the crystallike structures containing the protoxin are released in the larval gut, where the digestive enzymes cleave the protoxin, producing an active toxin.

Protoxin is activated within its gut by the combination of alkaline pH (7.5-8.0) and specific digestive proteases. The toxin binds to the membrane of the epithelium cells of the gut, inserts itself into that membrane and creates an ion channel through which other molecules (e.g., A TP) can freely pass. Punctured by many holes, the gut cells cannot survive long, so the insect larvae starve for lack of nutrition and ultimately die. Because conversion of the protoxin to the active toxin requires both alkaline pH and the presence of specific proteases, such conditions are not present in mammals and hence they are safe from the protoxin.

No significant role for the bacterium has been attributed to the parasporal crystal structure. The parasporal crystal usually consists mainly of protein (95%) and small amount of carbohydrate (5%). The crystal protein can generally be dissociated by mild alkali treatment into subunits. The insecticidal toxins of B. thuringiensis strains can be grouped into four major classes: Cry I, Cry II, Cry III and Cry IV. This is based on insecticidal activity against various insects (see Table 3.). These toxins are further classified in sub-classes and sub-groups according to DNA sequence of the toxin gene, e.g., Cry I gene has six sub-classes (Cry I A to F) and Cry I A has subgroups (Cry IA a to c).

As a result of co-evolution between insects and their pathogens, there is host specificity between Bt-toxin and the membranes of the gut cells. The Bt toxin of a particular B. thuringiensis strain will bind to the gut of lepidoptera larvae, or only some species of lepidoptera, but not to others. When toxin does not bind, there is no effect on the cells that line the gut, and the larvae do not die. Thus some Bt toxins will kill lepidoptera (butter flies and moths), others coleoptera (beetles and weevils) and others diptera (mosquitos). For the biological control of insect pests, approximately 1.3 x 108 to 2.6 X 108 spores per square foot of the target area is applied. Administration of the spores is timed to coincide with the peak of the larval population of the target organism.

B. thuringiensis subspecies kurstaki contains a protoxin gene on one of seven different plasmids that is approximately 2.0, 7.4, 7.8, 8.2, 14.4, 45 and 71kb in length. Protoxin is 130Kda, therefore, not present on small plasmids. This gene has been transferred to other bacteria to kill mosquito larvae as well as gene has been modified to produce toxin during vegetative phase of bacterial growth rather than only during sporulation. Thus, it is possible to produce toxin continuously in fermentor by growing bacteria. It has also been attempted to increase the host range.

This Bt toxin has been used in several ways to control the insects. A relatively simple way is to grow the B. thuringiensis bacteria, dry them out, and prepare the heat killed and dried bacteria in such a way that they can be sprayed or dusted on crops. These preparations are initially highly effective, but the Bt protoxin is not stable after product is sprayed on plants. The Bt protoxin crystals are released from the bacteria and protoxin quickly disappears from the plants.

Scientists at Mycogen, a biotechnology company in San Diego, California (USA), introduced a Bt gene in a different bacterium (Pseudomonas fluorescens). These bacteria can readily be grown in large fermentors, killed and than formulated as a spray. With this bacterium, the protoxin crystals remain in the bacterial cells, and as a result they are stable even after they have been sprayed on the plants. The spraying Bt toxin works well with insect larvae that live on the surfaces of leaves, but would be less effective with insect larvae that live in soil or larvae living inside the p1ants. To control these insects, scientists have transferred Bt gene using particle gun transfer system in cotton, tomato, tobacco, potatoes, and other crop plants. Transgenic plants produced containing Bt gene are listed in Table 4.

Table 4. Plants with insecticidal gene derived from B. thuringiensis

Company		*Transgenic crop*
Monsanto	—	Potato, cotton, tomato, corn
Calgene	—	Cotton, tobacco, potato,
Ciba-geigy	—	Tobacco, corn
Agrigenetics	—	Canola (rape seed)
Campbell Institute	—	Tomato
Rohm & Hass	—	Tobacco

Engineering for Antibiotic Resistance

Transgenic rice and maize plants have been regenerated by somatic embryogenesis from cell suspension-derived protoplasts electroporated with plasmid carrying the NPT II gene under control of the 35s promoter from CaMV. Heat-shock treatment of freshly isolated protoplasts prior to electroporatlon proved stimulatory for enhancing protoplast division and increasing the number of developed kanamycin-resistant colonies following exposure of protoplast-derived transformed cells to the antibiotic in the medium (Zhang et al. 1988, Rhodes et al. 1989). This could provide an opportunity for assessing the possibilities of introducing novel (non-selectable) genes into the economically important cereals. Similarly, transgenic plants of Petunia, tobacco, tomato, Arabidopsis and Brassica napus have been generated using a marker system AAC(3) coding sequences fused to the CaMV 35s promoter that confers resistance to gentamicin (Klee and Rogers 1989).

Conclusion

In conclusion, the recent progress made in the field of DNA transfer technology has renewed interest in plant genetic manipulations using this non-conventional procedure. Also, it has enabled a study of gene regulation at the molecular level. Through direct gene transfer, or Agrobacterium-mediated gene transfer, the expression of foreign genes has been observed in some important agricultural crops. Several of these can be stably transformed and mature plants regenerated. Genetic transformation systems can now be used to obtain transgenic plants of fibre (cotton), woody (Populus, Pinus and Pseudotsuga) and other crop species. It is likely that engineered plants will undergo field tests and become available on a commercial scale.

Questions

1. What are the methods by which you can obtain virus-resistant plants?
2. Give a brief account of herbicide resistant plants.
3. Write a short note on Bt gene.

Note